The Role of ATM (ataxia-telangiectasia mutated) in the mitochondrial pathway of apoptosis

Background – Ataxia-Telangiectasia

Ataxia-telangiectasia (AT) is a primary immunodeficiency disorder that occurs in an estimated 1 in 40,000 to 1 in 300,000 births (Leaderman, 2000). AT is characterized by progressive cerebellar ataxia, the loss of balance and coordination, oculocutaneous telangiectasia, the appearance of veins in the eyes that make them appear blood shot, progressive cerebellar dysfunction, and recurrent sinopulmonary infections secondary to progressive immunological and neurological dysfunction (Boder, 1958). AT patients are significantly predisposed to cancer, particularly lymphomas and leukemias. Other manifestations of the disease include sensitivity to radiation (Taylor et al., 1975), premature aging, and hypogonadism, the inadequate function of the testes or ovaries (Regueiro et al., 2000). AT has been a major interest of scientists since the 1960's because its many "different facets may yield clues about many other major health problems including neurological disease, cancer, immunodeficiency, and aging" (Leaderman, 2000).

The responsible gene in AT, ataxia-telangiectasia mutated (ATM), was discovered in 1995. Researchers had linked the hyper-sensitivity of AT patients to ionizing radiation (IR) and predisposition to cancer to "chromosomal instability, abnormalities in genetic recombination, and defective signaling to programmed cell death and several cell cycle checkpoints activated by DNA damage" (Canman, 1998). Earlier observations predicted that the gene altered in AT played a role in DNA damage recognition. These predictions were confirmed when a single gene on chromosome 11 was discovered by the combined efforts of Shiloh and colleagues (Savitsky et al., 1995, Gatti et al., 1982). Since its discovery, the protein product of the ATM gene has shown to be a part of "eukaryotic cell cycle control, DNA repair, and DNA recombination" (Zakian, 1995). Patients with AT have defects in their ATM gene, whereas those without AT have a normal copy.

Apoptosis is the process by which individual cells self-destruct when they become superfluous, disordered, or are a threat to the health of the organism, such as cells infected by viruses. Apoptosis is induced either through the death receptor pathway of apoptosis, or the mitochondrial pathway of apoptosis.

Objectives of the Project

Programmed cell death (apoptosis) is altered in AT (Takagi et al, 1998; Crompton et al., 1999; Liao et al, 1999); however the data on apoptosis in AT are limited and conflicting. Neither the pathways of apoptosis in AT nor the functions of ATM in lymphocyte apoptosis have yet been delineated. **Thus, the goal of this research project is to understand the role of ATM in apoptosis.** Looking at altered apoptosis arising from defective ATM genes would elucidate the role of ATM in apoptosis.

In formulating a workable hypothesis, we noted that in AT, DNA damage recognition is defective, and that DNA damage activates the mitochondrial pathway of apoptosis (Kroemer and Reed, 2000; Reed and Green, 2002). By this logic, we hypothesize that altered apoptosis in AT is via the mitochondrial pathway, and ATM plays a major role in maintenance of the mitochondrial micro-environment and signaling through the mitochondria.

Understanding the role of ATM in apoptosis would serve two purposes: It would elucidate involvement of the mitochondrial-pathway mediated apoptosis in immunopathogenesis of AT, which would offer basics to establish mitochondrion-targeted therapeutic interventions in AT, and would provide comprehensive information on the role of ATM in apoptosis and cellular functions, filling an important gap in this area of research. It would also serve as a model to investigate the pathogenesis of the nervous system damage.

Materials and Methods

We plan to investigate the changes in the mitochondrial micro-environment during apoptosis induced by an etoposide, an agent that causes double-strand breaks in DNA (Karpinich et al., 2002), in two model cell systems: B-Lymphoblastoid cells lines (lymphoid cells concerned with humoral immunity) from AT patients and control subjects, and primary fibroblast cell lines (skin cells) from AT patients and control subjects.

Our project consists of two specific parts:

Part 1: We will examine the apoptotic role of ATM in lymphoblasts and fibroblasts. To do this, we will study the time-kinetics of apoptosis induced by etoposide. Levels of apoptosis will be assayed by both PE-conjugated annexin-V staining with propidium iodide (PI) and TUNEL (*terminal deoxy-nucleotidyl transferase-mediated dUTP nick-end labeling*). Results from this section will set the stage for Part 2.

Part 2: We will examine the effect of ATM on mitochondrial micro-environment and pathway in apoptosis induced by etoposide by investigating each critical step in the mitochondrial pathway of apoptosis in both lymphoblast and fibroblast cell lines (same used in Part 1), using specific time point established from the data from Part 1. We will examine the following: (1)Mitochondrial membrane potential by TMRE staining, (2)Release of cytochrome c, AIF, and SMAC by immunocytochemistry using confocal microscopy, (3)Generation of reactive oxygen species by using hydroethidium staining, (4)Translocation of apoptotic Bax and anti-apoptotic Bcl-2 by western blotting, (5)Activation of caspase-9 and caspase-3 by colorimetric assay, and (6)PARP (poly ADP ribose polymerase) cleavage by flow cytometry. The results would clarify the regulation of apoptosis by ATM.

Predictions and Preliminary Studies

In Part 1, we expect that a defect in ATM (as in AT) would cause higher levels of etoposide-induced apoptosis in cells from AT patients compared to control patients. In Part 2, through the specific assays we expect to see a clear pattern of data that shows etoposide-induced apoptosis in AT in is both enhanced compared to control subjects and is via the mitochondrial pathway.

We have already established numerous lymphoblast and fibroblast cell lines, both with AT and control cells lines. With the lymphoblast cell lines, we have induced apoptosis with etoposide (100uM) in one control cell line and two AT cell lines. By TUNEL assay, both AT cell lines showed increased apoptosis compared to the controls (Fig. 1). We have also established the immunocytochemistry and fluorescence/confocal microscopic imaging for cytochrome c and AIF release (data not shown).

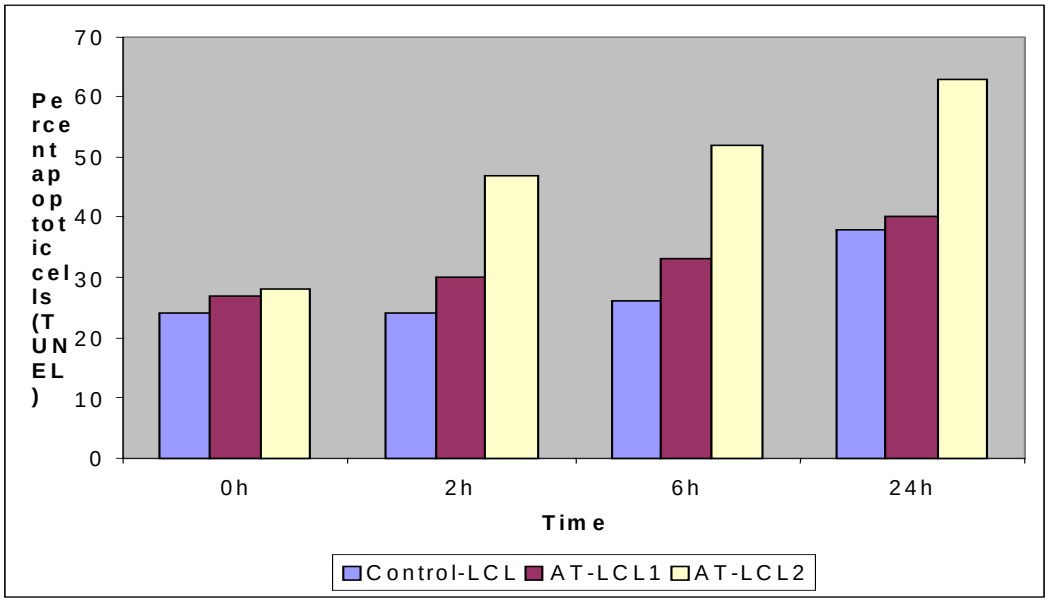

Figure 1. Etoposide induced apoptosis in one Control-LCL and two AT-LCLs (Etoposide 100uM).

Just recently, we set up 8 lymphoblast cell lines (6 AT and 2 control) and examined the levels of apoptosis over 120hrs with 0uM, 1uM, 10uM, 25uM, and 50uM concentrations of etoposide. We are in the process of reproducing the data.

My Specific Responsibilities & Timeline

I have been training for this position since winter, 2002. Working under my faculty mentor, and with the help of two other students, I have since become proficient in lab skills, setting up experiments, maintaining adherent fibroblast, and suspension lymphoblast cell lines, freezing cell lines, four color flow cytometry with Annexin-V, PI, and 7-AAD, caspase assays, and various other aspect of basic research. At the beginning of this school year, I was granted a degree of autonomy, which gave me the ability to set up and complete various experiments on my own, after consulting with my mentor.

My responsibilities include setting up and completing the aforementioned experiments and analyzing the data I find. As time progresses, I will learn more techniques and methods, of which I will put to use gathering data and completing parts of this project. More specifically, I will maintain specific cell lines that I will use to set up experiments, and will examine time kinetics, record and analyze data, and present my results to my mentor.

The project will take place over the course of one year. Part 1 will be completed by winter quarter, whereas part 2 will be completed by the end of summer. We have already set up the cell lines, and are in the process of gathering data for Part 1. Until the end of Part 1, we will continue to study the time-kinetics of etoposide-induced apoptosis in both lymphoblast and fibroblast cell lines. Once a sufficient amount of data and reproductions are achieved, we will move on to Part 2.

Part 2 will consist of examining different specific aspects of the mitochondrial micro-environment. Numerous assays will be conducted, as delineated in Materials and Methods, until all data is successfully collected and reproduced.

Budget

Item	Quantity	Price Each	Total Price
Apoptosis Detection Kits	2	$200	$400
RPMI Cell Culture Medium (500mL)	6	$50	$300
Fetal Bovine Serum (500mL)	1	$300	$300
		Total	**$1,000**

Justification

Since our project requires that we keep and maintain numerous cell lines, it is vital that we have enough cell medium. Cell medium in the lymphobast and fibroblast cell lines changed every 2-4 days. Because of this, we need substantial amounts of cell medium to maintain the cell lines. Both cell models need RPMI w/ 15-20% Fetal Bovine Serum. Six 500mL bottles of RPMI and one 500mL bottle of Fetal Bovine serum would allow us to maintain all cell lines long enough to complete part 1. Also, in Part 1, we will need to detect apoptosis in

each cell line at various concentrations of etoposide and at different time points. Therefore, two apoptosis detection kits are vital.

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